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Minireview

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Jessica L Whited and Clifford J Tabin

Address: Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.

Correspondence: Clifford J Tabin. Email: tabin@genetics.med.harvard.edu

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Abstract

The investigation of vertebrate limb regeneration, a favorite topic of early developmental biologists, is enjoying a renaissance thanks to recently developed molecular and genetic tools, as indicated in recent papers in *BMC Biology* and *BMC Developmental Biology*. Classical experiments provide a rich context for interpreting modern functional studies.

Nearly 240 years have passed since the first scientific treatise addressing limb regeneration, Spallanzani's 'Reproduction of the Legs in the Aquatic Salamander' within his *An Essay on Animal Reproductions* [1]. In spite of extraordinary advances in other areas of developmental biology in the past few decades, many of the most remarkable features of limb regeneration outlined by Spallanzani remain mysterious today. However, recent advances in genomics and molecular biology offer the potential to finally illuminate the cellular and molecular mechanisms underlying amphibian limb regeneration. Changes in gene expression accompanying regenerative events can now be profiled by microarrays. Recent projects by Monaghan *et al.* [2] published in *BMC Biology* and by Pearl *et al.* [3] in *BMC Developmental Biology* have provided thousands of cDNA sequences of transcripts expressed during limb regeneration in amphibians. Moreover, the newly developed application of transgenesis to axolotl salamanders [4] suggests that functional roles for specific genes are likely to be elucidated in the near future. As these tools are brought to bear on the problem of limb regeneration, work will build on and be guided by the extensive classical literature, including both experimental and descriptive studies.

Wound healing makes all the difference

Following amputation, a salamander's limb bleeds only briefly and the important operation of healing the wound in a way conducive to regeneration begins. Within 24 hours, the cut surface is ensheathed by epithelial cells that migrate from the surface of the stump (Figure 1). These 'wound epidermis' cells proliferate, forming the 'apical epidermal cap' (AEC), a structure postulated to provide key molecular signals needed to stimulate and/or maintain the early stages of regeneration. Without this specialized wound healing, regeneration fails; for instance, if the limb is amputated and the dorsal and ventral skin is pulled together and sutured, no true AEC forms and the limb remains a stump.

Building a blastema

The next critical step is to create a blastema - a pool of cells from which the new limb will arise. Forming at the distal tip of the old stump but beneath the AEC, the blastema morphologically appears as a transparent outgrowth that acquires the shape of a cone as regeneration proceeds (Figure 1). Blastema cells are thought to be relatively undifferentiated mesenchymal cells, but their origins remain

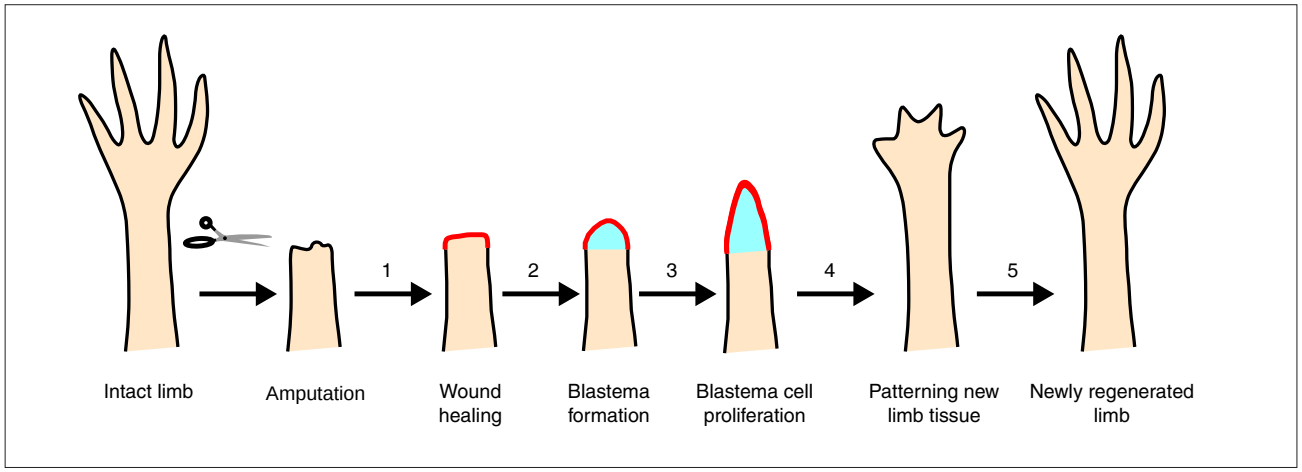

Figure 1
Key morphological events of vertebrate limb regeneration. Following amputation, epidermal cells from the surface of the stump rapidly migrate to cover the wound (1), forming the apical epidermal cap (AEC, red). Stump cells are used to create a blastema (blue) beneath the AEC (2). Blastema cells proliferate and the structure acquires a cone-shaped morphology (3). Undifferentiated blastema cells begin to differentiate into various cell-types within the newly formed limb (4). The new portion continues to grow. Once patterning and growth are complete, a perfectly functional new limb has been regenerated (5).

highly controversial (reviewed in [5]). Early work suggested that at least some blastema cells arise by the dedifferentiation of muscle fibers, as the fibers immediately adjacent to the amputation plane showed microscopic signs of cellularization, and these presumably newly created mononucleate cells incorporated tritiated thymidine [6]. Studies using modern labeling techniques, such as fluorescent dye tracking and fluorescently labeled antibodies, support a similar model, yet controversy remains because others claim that a stem-cell population, the muscle satellite cells, also participate in blastema formation. Furthermore, the possibility of transdifferentiation of cells in the stump to different cell types in the regenerate, a process hinted at in earlier studies, needs to be definitively addressed, both in terms of the potential of blastema cells for transdifferentiation and the extent to which this phenomenon is significant for normal regeneration. These questions await more sophisticated cell-lineage analysis. Such analysis may be facilitated by the identification of cell-type-specific promoters in conjunction with the recently developed transgenic approaches.

Once the blastema cells are collected under the AEC, they must proliferate to provide enough cells to drive the regeneration process forward (Figure 1). The proliferation of blastema cells has been shown to be critically reliant on the presence of the nerve in the limb [7]. For example, a limb that has been denervated and then amputated will close the wound in an outwardly normal manner, and a blastema will form, but the blastema cells do not proliferate enough and regeneration fails. Interestingly, if a limb is manipulated to develop originally without the nerve, this limb can be amputated and a fairly normally regenerated limb grows. These data suggest the limb somehow becomes 'addicted' to factors produced by the nerve and then needs them for regeneration.

Recent work has shown that regeneration of a denervated limb can be mostly rescued by providing cDNA encoding a single protein, nAG [8]. nAG is a secreted ligand for Prod1, a hitherto mysterious cell-surface molecule whose expression is graded along the proximal-distal axis in a salamander limb. A yeast two-hybrid strategy was used to uncover nAG, and the relatively modern technique of electroporation of plasmid DNA into limb blastemas was used to demonstrate its sufficiency for replacing the nerve.

While the outlines of blastema formation are fairly well understood, relatively few molecules have been implicated in specific events that form and shape the blastema. Much work remains to discover the cellular origins of blastema cells, how these cells are cued to form a blastema, and how the blastema cells are stimulated to proliferate. Some clues may be found using genomic approaches, as shown by the recent study by Monaghan *et al*. [2], where many transcripts were identified as differentially expressed in blastemas undergoing normal regeneration compared with those whose limb had been denervated.

Finishing the job

Eventually, blastema cells begin the process of reorganizing and of specifying distinct cellular identities for the new limb. Morphologically, the blastema becomes flattened and acquires the shape characteristic of a 'palette-staged' limb bud with the vague outline of future digits discernable (Figure 1). Most of the events governing the regeneration process from this point onward are presumed to be similar or identical to the molecular events that transform a limb bud into a limb. It is, however, important to note that many of these assumptions remain to be tested, and that the two scenarios cannot be completely equivalent. For instance, the scale at which a limb regenerates is often many times - perhaps even thousands of times - larger than that at which it developed when the animal was a tiny larva. In addition, new features such as blood vessels and fine nerves need to be seamlessly integrated into the existing structures on the stump if the limb is to thrive and function properly. Nonetheless, some mechanisms have already been shown to be common; for example, the ectopic production of Sonic hedgehog signaling activity in the anterior margin of a regenerating limb produces the same effect - duplication of posterior digits - in a regenerating blastema as in a newly developing limb bud [9].

Decoding the secrets of perfect regeneration

If all steps proceed normally, the salamander or tadpole regrows a perfect replica of its original limb. This precise replication is one of the most remarkable aspects of regeneration. An animal that loses a foot will grow back only a foot and no more; one that loses the leg from the thigh will grow back everything that was once distal to the thigh's amputation plane. Somehow, the salamander's body can measure where the amputation occurred along the proximal-distal axis and replace only the missing part, but how?

While the process is still poorly understood, some clues have come from blastema-grafting experiments (reviewed in [10]). When grafted to a proximal 'thigh' blastema, a distal blastema 'fated' to make a foot translocates distally with the host's regenerating limb and gives rise to a regenerated limb that essentially has two feet. Alternatively, a proximal blastema grafted to a proximal blastema host will create a salamander with essentially two complete legs. Therefore, the proximo-distal information is encoded within the blastema. Remarkably, if a proximal limb blastema is grafted to a receptive field such as the eye (parts of which can also regenerate in many salamanders), a limb will grow from the eye socket, demonstrating that the blastema is indeed an autonomous unit and, once created, may only rely on the underlying tissue for survival factors but not for contextual information. On a molecular level, there is evidence that the cell surface protein Prod1, mentioned above, plays a critical role in mediating proximo-distal positional information. However, the question of how positional information is established in the blastema and how it influences cell behavior to achieve precise replacement of amputated structures remains largely untouched but will benefit from the application of the modern genomic and genetic techniques discussed earlier.

Understanding the molecular and cellular mechanisms that allow salamanders to create and develop a blastema may help develop therapies for improving regeneration in animals that do not. A good starting point for comparison is a salamander, which can regenerate throughout its life, and a frog, which can only regenerate limbs while it is a tadpole and gradually loses the ability to regenerate as it approaches the final step of metamorphosis. An even simpler comparison can be made between a tadpole at a stage that regenerates versus a later-staged tadpole that cannot. The recent work from Caroline Beck's lab (Pearl *et al*. [3]) profiled gene expression in blastemas from normally regenerating tadpoles compared with those in which regeneration was blocked by the misexpression of Noggin, an inhibitor of the secreted signal molecule bone morphogenetic protein (BMP). Genes defined as essential regulators of regeneration in this case included those that specifically influence the transition from an early blastema to a larger, cone-shaped blastema (the step that is blocked in the absence of BMP activity).

Similar approaches may prove fruitful for discovering transcripts expressed at other discrete stages, for instance, during the critical wound healing that initiates limb regeneration in the salamander. Further evidence for the importance of this step comes from human medicine: in young children with distal amputations of digits, regeneration of a perfect fingertip can occur, but only if the stump skin is not sutured together. If early healing stages were better understood in both regenerating and non-regenerating scenarios, we would have a better chance of figuring out how to heal a wound in a way that leads to formation of a blastema.

Regeneration research is now undergoing a resurgence, with initial efforts fueled by modern approaches to understanding gene expression. Upcoming work will take advantage of the power of transgenesis to explicitly address the functions of specific genes at particular stages of regeneration and in particular cell types. Additional tools are still needed, however. Limb regeneration is most impressive among salamanders, and no salamander genomes have been sequenced to date (mostly due to their enormous size). Moreover, a reliable method for eliminating or reducing gene function in salamanders has not yet been established.

As such new genetic and genomic tools are developed, we will be able to fully realize the power of salamanders as model systems for understanding limb regeneration.

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References

1. Spallanzani A: **Reproductions of the legs in the aquatic salamander.** In *An Essay on Animal Reproductions*. London: Becket and de Hondt; 1769: 68-82.
2. Monaghan JR, Epp LG, Putta, S, Page RB, Walker JA, Beachy CK, Zhu W, Pao GM, Verma IM, Hunter T, Bryant SV, Gardiner DM, Harkins TT, Voss, SR: **Microarray and cDNA sequence analysis of transcription during nerve-dependent limb regeneration.** *BMC Biol* 2009, **7:**1.
3. Pearl EJ, Barker D, Day RC, Beck CW: **Identification of genes associated with regenerative success of *Xenopus laevis* hindlimbs.** *BMC Dev Biol* 2008, **8:**66-81.
4. Sobkow L, Epperlein HH, Herklotz S, Straube WL, Tanaka EM: **A germline GFP transgenic axolotl and its use to track cell fate: dual origin of the fin mesenchyme during development and the fate of blood cells during regeneration.** *Dev Biol* 2006, **290:**386-397.
5. Bryant SV, Endo T, Gardiner DM: **Vertebrate limb regeneration and the origin of limb stem cells.** *Int J Dev Biol* 2002, **46:**887-896.
6. Hay ED, Fischman DA: **Origin of the blastema in regenerating limbs of the newt *Triturus viridescens*. An autoradiographic study using tritiated thymidine to follow cell proliferation and migration.** *Dev Biol* 1961, **3:**26-59.
7. Brockes JP: **The nerve dependence of amphibian limb regeneration.** *J Exp Biol* 1987, **132:**79-91.
8. Kumar A, Godwin JW, Gates PB, Garza-Garcia AA, Brockes JP: **Molecular basis for the nerve dependence of limb regeneration in an adult vertebrate.** *Science* 2007, **318:**772-777.
9. Roy S, Gardiner DM, Bryant SV: **Vaccinia as a tool for functional analysis in regenerating limbs: ectopic expression of *shh*.** *Dev Biol* 2000, **218:**199-205.
10. Nye HLD, Cameron JA, Chernoff EAG, Stocum DL: **Regeneration of the urodele limb: a review.** *Dev Dyn* 2002, **226:**280-294.